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Papillomavirus infections – a major cause of human cancers

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1. Summary

The papillomavirus family represents a remarkably heterogeneous group of viruses. At present, 77 distinct genotypes have been identified in humans and partial sequences have been obtained from more than 30 putative novel genotypes. Geographic differences in base composition of individual genotypes are generally small and suggest a low mutation rate and thus an ancient origin of today's prototypes. The relatively small size of the genome permitted an analysis of individual gene functions and of interactions of viral proteins with host cell components. Proliferating cells contain the viral genome in a latent form, large scale viral DNA replication, as well as translation and functional activity of late viral proteins, and viral particle assembly are restricted to differentiating layers of skin and mucosa.

In humans papillomavirus infections cause a variety of benign proliferations: warts, epithelial cysts, intraepithelial neoplasias, anogenital, oro-laryngeal and -pharyngeal papillomas, keratoacanthomas and other types of hyperkeratoses. Their involvement in the etiology of some major human cancers is of particular interest: specific types (HPV 16, 18 and several others) have been identified as causative agents of at least 90% of cancers of the cervix and are also linked to more than 50% of other anogenital cancers. These HPV types are considered as '*high risk*' infections. Their E6/E7 oncoproteins stimulate cell proliferation by activating cyclins *E* and *A*, and interfere with the functions of the cellular proteins *RB* and *p53*. The latter interaction appears to be responsible for their mutagenic and aneuploidizing activity as an underlying principle for the progression of these HPV-containing lesions and the role of high risk HPV types as *solitary carcinogens*. In non-transformed human keratinocytes transcription and function of viral oncoproteins is controlled by intercellular and intracellular signalling cascades, their interruption emerges as a precondition for immortalization and malignant growth.

Recently, novel and known HPV types have also been identified in a high percentage of non-melanoma skin cancers (basal and squamous cell carcinomas). Similar to observations in patients with a rare hereditary condition, *epidermodysplasia verruciformis*, characterized by an extensive verrucosis and development of skin cancer, basal and squamous cell carcinomas develop preferentially in light-exposed sites. This could suggest an interaction between a physical carcinogen (UV-part of the sunlight) and a '*low-risk*' (non-mutagenic) papillomavirus infection. Reports on the presence of HPV infections in cancers of the oral cavity, the larynx, and the esophagus further emphasize the importance of this virus group as proven and suspected human carcinogens.

2. Introduction

The infectious nature of human and animal warts was demonstrated at the turn of this century (reviewed in [1]). First experimental attempts to relate these infections to cancer development and to study interactions with other carcinogenic factors were made by Rous and his associates in the 1930th and in the subsequent two decades [2–5]. Based on initial observations by [6], Rous demonstrated in ingenious experiments the carcinogenic potential of a cottontail rabbit papillomavirus infection in domestic rabbits and syncarcinogenic activity of tar and of defined chemical carcinogens, when jointly applied with the virus infection. The carcinogenic activity of the Shope papillomavirus (later renamed *cottontail rabbit papillomavirus* or *CRPV*) was subsequently irrefutably proven by [7], who induced carcinomas in domestic rabbits with purified CRPV DNA or with DNA extracted from CRPV-induced papillomas and carcinomas.

The first visualisation of papillomavirus particles in human warts by electronmicroscopy was reported in 1949 [8]. The structure of papillomavirus genomes was unravelled by Crawford and Crawford in 1963 [379]. The unavailability of tissue culture systems, however, and the apparent benign nature of human warts led to few additional experimental approaches in subsequent years.

Almost unnoticed by contemporary tumor virology, blossoming in the late 1950s and 1960s due to the discovery of murine leukemia viruses [9] and the DNA tumor viruses polyoma [10], SV40 [11], and adenovirus type 12 [12], two different lines of studies contributed to the development of papillomavirus research: In 1959 Olson and colleagues [396] reported the induction of urinary bladder tumors in cattle by a bovine papillomavirus found in skin fibropapillomas. The same virus turned out to be tumorigenic in hamsters [13,14] and transformed calf and

murine cells in tissue culture [15,16]. Thus, a second member of the papillomavirus group, besides CRPV, was clearly able to induce malignant tumors.

The second study roots back to 1922, when Lewandowsky and Lutz [392] reported a rare and obviously hereditary generalized verrucosis in humans with skin carcinoma development at sun-exposed sites. They labeled this syndrome *Epidermodysplasia verruciformis* and were not aware at that time of the potential infectious origin of the papillomatous plaques and macules covering the affected skin. This was subsequently demonstrated by [17] and by Jablonska and her colleagues [18,19] after inducing papillomas following intracutaneous autoinoculation of cell-free extracts. Jablonska realized in 1972 that this condition could serve '*as model in studies on the role of papovaviruses in oncogenesis*'.

Gradually interest in papillomaviruses evolved in the second part of the 1970s, evidenced by the first papillomavirus workshops, commonly attended at that time by 15 to 30 participants. This developed in part from the hypothesis that papillomaviruses may play a significant role in the etiology of cancer of the cervix [20,21]. Tests to substantiate this hypothesis had established the plurality of papillomavirus types and subtypes [22–24]. In addition, Meisels and Fortin [395] proposed a papillomavirus origin of koilocytotic atypias, separating them from 'true' preneoplastic lesions. This promised to represent a valuable diagnostic aid in grading lesions for surgical intervention. The demonstration of papillomavirus particles in typical koilocytes underlined their observations [25–27]. Papillomavirus research, however, was also stimulated by the identification of novel HPV types in lesions of patients with epidermodysplasia verruciformis (EV), and here particularly in malignant tumors of such patients [24,28].

In the 1980s the situation changed almost abruptly: the isolation of new HPV types (HPV 6 and 11) from genital warts [29,30], and subsequently directly from cervical cancer biopsies HPV types 16 and 18 [31,32] resulted in a rapid expansion of experimental work and also in early epidemiological approaches. In spite of numerous efforts from various laboratories, it took almost one decade before the causal role of specific HPV types in cancer of the cervix and the respective precursor lesions was more or less generally accepted [199,33–35].

Today the main interest shifted to mechanisms of carcinogenesis by papillomaviruses: how do genes of these viruses influence cell growth, how do their oncoproteins interact with host cell components, and to which extent is the failure of specific host cell functions related to papillomavirus-induced oncogenesis? The very recent recognition of a linkage of other widespread human tumor types, like cancers of the skin and of the oropharynx, with HPV infections points to the magnitude of the problem. Papillomaviruses emerge as the most common *carcinoma viruses* [36] and appear to play a 'secret' role as major cancer pathogens [37]. Successful first attempts to vaccinate animals against their own papillomavirus infections [377], raise at the same time the hope for the prevention of specific human cancers based on similar vaccination protocols.

This review tries to summarize our present understanding of papillomavirus infections by emphasizing their role in human cancers and by analyzing specific aspects of their interaction with the infected host and specific host cell components. For a detailed description of the molecular biology of papillomaviruses and of immunological and epidemiological aspects the reader is referred to a number of other reviews [402;38,384,219,388].

3. Structure of viral particles and taxonomy

3.1. Viral particles and late proteins

The diameter of papillomavirus (HPV) particles amounts to approx. 55 nm. Full particles contain the double-stranded closed circular DNA genome. The viral DNA is associated with histone-like proteins [39,40] and encapsidated by 72 capsomeres [41]. The major capsid protein is coded for by the L1 open reading frame, it seems to contain reactive epitopes for type-specific neutralization. The L2 open reading frame codes for an additional structural component of the viral capsid. Antigenic domains of this protein appear to be responsible for a group-specific reactivity of antisera. Virus-like particles, containing the structural components of various types of HPV can, however, be obtained by the expression of these proteins in recombinant vectors [42,43], obviously the L1 protein suffices for this particle formation. This protein has a mol wt. of approx. 55 000 and is highly conserved among different papillomavirus types. The second structural protein, L2, is less conserved and possesses a molecular weight of about 75 000. The non-enveloped structure renders papillomaviruses relatively resistant to heating and to organic solvents [44].

3.2. Structure and regulation of the viral genome

The genome consists of 7200–8000 base pairs of closed-circular double-stranded DNA containing up to 10 open reading frames (Fig. 1). The structure of the viral genomes reveals remarkable similarities between different members of this virus group: generally only one strand is transcriptionally active, therefore transcription occurs in one direction only, and the localization of open reading frames reveals a remarkable degree of correspondence [45,46]. Papillomavirus genomes can be divided into three regions: a long control region (LCR) covering about 10% of the genome, and early (E) and a late (L) region. The L genes code for structural proteins, the E region mainly for regulatory functions engaged in genome persistence, DNA replication, and activation of the lytic cycle.

The regulation of viral gene expression is complex and controlled by cellular and viral transcription factors. Most

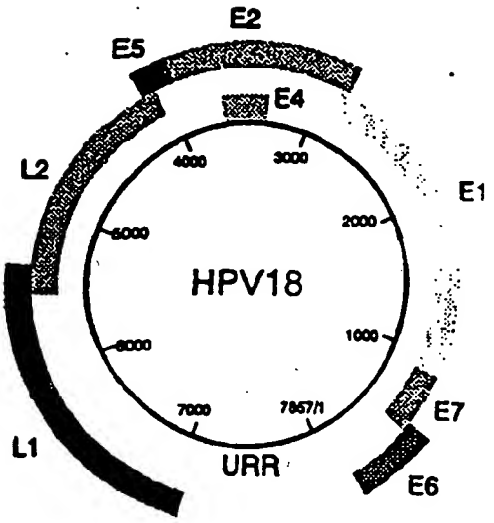

Fig. 1. Circular map of the HPV18 genome indicating the localization of open reading frames (E1 to E7 for early proteins, L1 and L2 for late proteins) and the upstream regulatory region (*URR* or long control region, *LCR*). The figure was kindly provided by Dr. Felix Hoppe-Seyler.

of these regulations occur within the LCR region which varies substantially in nucleotide composition between individual HPV types. The LCRs of anogenital HPVs range in size between 800–900 bp, in other papillomaviruses, particularly in those found in EV-lesions, they are somewhat shorter. Within the LCR *cis*-active elements regulate transcription of the E6/E7 genes which represent the transforming genes for immortalization and for the maintenance of the malignant phenotype of HPV-positive cervical cancer cells [47–49].

A large number of cellular transcription factors have been identified, binding to the frequently studied HPV18 LCR: among them NF-1, API, KRF-1, Oct-1, SP-1, YY-1, and the glucocorticoid receptor [50–52,409,54, 410,411,55,56]. The dysfunction of some of them appears to play a significant role in papillomavirus-linked carcinogenesis [57]. Very recently a novel epithelial factor, Epoc-1/Skn-1a, was identified, regulating papillomavirus transcription differentiation-dependent in suprabasal cells [58]. Most of these factors bind to the central region of the LCR, the *enhancer*-region. They regulate the transcription of the E6/E7 promoter located at the 3'-terminus of the LCR. Although many of these factors stimulate the promoter, some of them (specifically YY-1) have a dual function and repress and stimulate the viral promoter [56,59,282].

Besides the cellular regulation, intragenomic regulation of the E6/E7 gene activity by the viral E2 protein plays an important role in activating or suppressing these oncogenes (see below). The LCR of HPV 16 contains four E2 binding sites. The 5'-distal part of the LCR contains the first E2 binding site, forming the terminal part of this region, and the translation termination codon for L1. The same segment harbours transcription termination and polyadenylation sites for late transcripts and acts as a negative regulator at the level of late mRNA stability [60,61].

The central segment of the LCR is flanked by two E2 binding sites. The E2 binding site at the 3' part is engaged in replication initiation, but also modulates E6/E7 transcription [55]. The proximal segment contains the promoter region, terminated 5' by the E2 site and 3' by the translation start codon of the E6 gene. Two additional E2 binding sites are located within the proximal 90 base pairs overlapping a TATA box. E2 binding of these sites modulates the promoter activity by displacing the basic transcription complex [381,62].

3.3. Nomenclature and taxonomy

Since 1976 [22], the genetic heterogeneity of the human papillomavirus group became more and more apparent. In 1978 a small conference took place in Mobile, Alabama, resulting in a proposal for HPV nomenclature: it was decided to designate new types if they differed by more than 50% from known prototypes when tested by reassociation kinetics performed under stringent conditions of hybridization [63]. When more and more sequence data became available, it was decided on one of the subsequent workshops to use DNA comparisons of the E6, E7, and L1 open reading frames for typing of HPVs [64]. An overall difference within these open reading frames of more than 10% was used to define new types. At the Annual Papillomavirus Conference 1995 in Quebec City this issue was reconsidered. Differences in only the L1 open reading frame exceeding 10% from established prototypes were used from now on for the definition of new types.

To date 77 distinct HPV genotypes have been described and the genomic sequences of most of them have been fully analyzed [65,380]. They are listed in Table 1. About 30 additional partial sequences have been obtained of putative novel HPV types, suggesting that the total number of existing HPV genotypes exceeds well 100. The explosion in identifying novel types originates from technical advances, particularly from the application of PCR technology. The arbitrary definition of novel types, a difference of more than 10% in the nucleotide sequences in the E6, E7, and L1 open reading frames, seems to define natural taxonomic units, since most recent isolates represented either novel types or were identical or differed only marginally from established prototypes [66]. In spite of the enormous heterogeneity of this virus group, mutational changes appear to occur at low frequency, indicating diversification of the types already in prehistoric times.

Based on their nucleic acid composition, a number of papillomavirus subgroups can be defined [67]. One of the largest known subgroup is represented by HPV types infecting mainly mucosal surfaces, most frequently of the anogenital tract. More than 40 of the identified HPV types belong into this group. The best known prototype of this

Table 1
Characterized HPV types (from [64,65], and unpublished data)

HPV type	Preferentially found in:
1	plantar warts
2	common warts
3	flat warts
4	common warts
5	benign and malignant EV lesions
6	genital warts, laryngeal papillomatosis
7	'butcher's' warts, oral papillomas of HIV patients
8	benign and malignant EV lesions
9	EV lesions
10	flat warts
11	laryngeal papillomas, genital warts
12	EV lesions
13	oral focal epithelial hyperplasia
14	EV lesions
15	EV lesions
16	anogenital intraepithelial neoplasias and cancers
17	EV lesions
18	anogenital intraepithelial neoplasias and cancers
19	EV lesions
20	EV lesions
21	EV lesions
22	EV lesion
23	EV lesions
24	EV lesions
25	EV lesions
26	common warts under immunosuppression
27	common warts
28	flat wart
29	common wart
30	laryngeal carcinoma
31	anogenital intraepithelial neoplasias and cancers
32	oral focal epithelial hyperplasia, oral papillomas
33	anogenital intraepithelial neoplasias and cancers
34	anogenital intraepithelial neoplasias
35	anogenital neoplasias and cancers
36	actinic keratosis, EV lesions
37	keratoacanthoma *
38	melanoma *
39	anogenital intraepithelial neoplasias and cancers
40	anogenital intraepithelial neoplasias
41	cutaneous squamous cell carcinomas
42	anogenital intraepithelial neoplasias
43	anogenital intraepithelial neoplasias
44	anogenital intraepithelial neoplasias
45	anogenital intraepithelial neoplasias and cancers
46 **	EV lesions
47	EV lesions
48	cutaneous squamous cell carcinoma
49	flat wart under immunosuppression
50	EV lesion
51	anogenital intraepithelial neoplasias and cancers
52	anogenital intraepithelial neoplasias and cancers
53	anogenital intraepithelial neoplasias
54	anogenital intraepithelial neoplasias
55	anogenital intraepithelial neoplasia
56	anogenital intraepithelial neoplasias and cancers
57	oral papillomas and inverted maxillary sinus papilloma
58	anogenital intraepithelial neoplasias and cancers
59	anogenital intraepithelila neoplasias
60	epidermoid cysts
61	anogenital intraepithelial neoplasias
62	anogenital intraepithelial neoplasias
63	myrmecia wart
64	anogenital intraepithelila neoplasia
65	pigmented wart
66	cervical carcinoma
67	anogenital intraepithelial neoplasia
68	anogenital intraepithelial neoplasia
69	anogenital intraepithelial neoplasias and cancers
70	vulvar papilloma
71	anigenital intraepithelila neoplasia
72	oral papilloma (HIV patient)
73	oral papilloma (HIV patient)
74	anogenital intraepithelial neoplasia
75	common wart in organ allograft recipient
76	common wart in organ allograft recipient
77	common wart in organ allograft recipient

* Only individual isolates.
** Now designated HPV 20b.

group is HPV16. Another subgroup is represented by viruses found in epidermodysplasia verruciformis lesions, with HPV5 as the most prominent member. These viruses are also found in lesions of patients suffering from immunosuppression. A third subgroup contains a few virus types preferentially found in cutaneous lesions. The prototype of this subgroup is HPV 4. A fourth subgroup finally is in itself heterogeneous and contains some distantly related viruses like HPV1, HPV63, and HPV41.

The heterogeneity of the human papillomavirus group is not restricted to the human members, the large number here seems to reflect the intensity of investigations. Thus far 8 bovine papillomavirus types have been isolated. Four types have been cloned from monkeys and apes.

It is interesting to note that a number of animal papillomaviruses are more closely related to individual members of the human subgroups than the latter among each other. A rhesus monkey papillomavirus isolated from a penile carcinoma of these monkeys [68] is very closely related to HPV 52. Similarly, there exists a very close relationship between a pygmy chimpanzee papillomavirus and HPV13 [69]. The cottontail rabbit papillomavirus and the canine oral papillomavirus belongs into the subgroup of HPV1, 63, and 41 [70,71]. These observations stress the assumption of the development of the papillomavirus group far back in prehistoric times.

4. Functions of viral proteins

4.1. E2 protein

The E2 open reading frame encodes at least two and probably three different proteins, all acting as transcription factors [72]. They differently affect viral gene expression and represent major intragenomic regulators by forming

dimers at specific binding sites. HPV16 and HPV 18 E2 protein function as transcriptional activators in human cervical keratinocytes [72–74]. The C-terminal domain of the HPV 16 E2 gene acts as transcriptional repressor and interferes with the activity of the full length E2 protein [75].

Deletion of the E2 open reading frame is frequently observed in cervical cancer biopsies and in cell lines derived from this cancer [76], leading to the speculation that this deletion facilitates transformation of human cells and the transition into a malignant state. Indeed, mutations in the E2 ORF, but also in the E2 DNA binding sites within the viral LCR led to enhanced immortalizing activity of HPV 16 DNA [77]. In cancer development, disruption of E2 appears, however, to usually represent a late event since most premalignant lesions do not reveal this modification [78,79]. A recent study noted integration of HPV 16 DNA also in advanced cervical intraepithelial neoplasias [80]. Besides its role in transcriptional regulation, E2 proteins interacting with E1 stimulate viral DNA replication [81–83]. They apparently facilitate binding of E1 to the origin of replication [84].

4.2. E1 protein

E1 shares a number of properties with SV40 large T antigen [84,85]: It codes for a policistronic RNA, the protein has site-specific DNA binding functions [86], binds and hydrolyzes ATP [84], possesses ATP-dependent helicase activity [87] and is essential for papillomavirus replication [88]. It also interacts with cellular DNA polymerase α [407]. The E1 protein binding site in the origin of replication, localized in the proximal region of the LCR, represents an 18 nucleotides imperfect palindrome [89]. Bidirectional unwinding of this region is a prerequisite for viral DNA replication [90]. Besides L1, the E1 open reading frame represents the most conserved structure among different papillomavirus types.

4.3. E5 protein

The E5 protein is the major transforming protein in bovine papillomaviruses [91–93]. In contrast, in HPV infections E5 has only weak transforming activity [94–96]. It may cause tumorigenic transformation of mouse keratinocytes, leads to anchorage-independent growth of mouse fibroblasts, and stimulates growth of primary rat kidney epithelial cells in cooperation with the HPV16 E7 gene [97,72]. The open reading frame coding for E5 is frequently deleted in cervical cancers [76], although anogenital low grade intraepithelial neoplasias contain relatively large amounts of E5 mRNA and protein [98,99]. This may support the assumption that E5 plays a role in early steps of HPV infection but is obviously dispensable for the maintenance of malignant transformation.

The hydrophobic E5 protein is mainly localized within the Golgi apparatus, in part it is also found in the plasma membranes [100]. BPV1 E5 protein binds and enhances the effect of platelet-derived growth factor (PDGF) and of epidermal growth factor (EGF), an effect not seen by HPV 16 E5 [101–103]. The latter E5 protein, however, reduced the degradation of internalized EGF receptors. Recent studies demonstrate complex formation between HPV 16 E5 protein with epidermal growth factor receptor, platelet-derived growth factor β receptor, colony stimulating factor-1 receptor, and with vesicular stomatitis virus glycoprotein [104]. Thus, this protein complexes with a variety of other transmembrane proteins. BPV1 and HPV16 E5 proteins also associate with the membrane-bound proton-ATPase which is part of the gap-junction complex [105,383]. In HPV16 E5-transfected cells a strong impairment of microinjected Lucifer yellow was noted, correlating with dephosphorylation of connexin 43, a major gap junctional protein [106].

4.4. E4 protein

The E4 protein seems to be incorrectly assigned as an early gene product. It originates from a viral RNA transcript formed by a single splice between the beginning of the E1 open reading frame and the E4 open reading frame. This mRNA is the major transcript in HPV-induced lesions [107,108]. The role of this protein in the life cycle of the virus has yet to be determined. It is not required for transformation or episomal persistence of viral DNA [109]. The E4 protein is exclusively localized within the differentiating layer of the infected epithelium [110,111,325,397]. It has been speculated that this protein plays a role in productive infection, possibly by disrupting normal differentiation, establishing favorable conditions for viral maturation

E4 proteins associate with the keratin cytoskeleton of cultured epithelial cells [112,113]. Electron microscopically they can be localized to tonofilament-like structures in HPV1 warts [412]. HPV16 E4 induces a collapse of the cytokeratin network in cultured cells [112,113]. Multiple E4 proteins have been demonstrated in HPV1-infected cells [110]. This may result from differential expression but also from posttranslational modifications and should influence the functional activity of E4 proteins [114].

Even papillomavirus types sharing tissue specificity reveal only limited homology in DNA sequences coding for E4 proteins [115]. The HPV1 E4 protein has been identified as a zinc finger protein [116]. The functional consequences of this property are presently unknown.

4.5. E6 and E7 proteins

E6 and E7 proteins are expressed in HPV-positive cancer cells These proteins may cause immortalization of

human keratinocytes and of a number of other cell types (see Section 4). Those HPV types coding for E6 and E7 genes involved in immortalization of tissue culture cells and found frequently in malignant tumors, are considered as *high risk* HPVs, contrasting an apparently low tumorigenic potential of other types, generally designated as *low risk* HPVs [117]. E6, and E7 genes code for growth-stimulating proteins, particularly E6 and E7 of specific types are relevant for the progression to malignant growth (reviewed in [118]).

Both proteins of high risk types cooperate in immortalization and transformation [408,47]. E7 proteins of these viruses, however, are able to transform established rodent cell lines by themselves (reviewed in [119]. Similarly E6 represents an independent oncogene since it immortalizes human mammary epithelial cells [120].

4.5.1. E6 protein

The E6 protein of HPV 16 contains 151 amino acids and reveals four Cys-X-X-Cys motifs mediating zinc-binding which may result in the formation of two zinc finger structures [121–123]. The E6 protein of high risk HPVs possesses a number of interesting biological properties: it cooperates with the E7 protein in the immortalization of human cells [47]. Introduction of this gene into specific types of human mammary cells may lead to immortalization even in the absence of E7 [120,124]. E6 of these virus types, in addition, cooperates with the *ras* oncogene in the immortalization of primary rodent cells [125] and induces anchorage-independent growth of NIH 3T3 cells and transcriptionally transactivates the adenovirus E2 promoter [126].

A most significant observation related to the function of the E6 protein was made initially by [127], revealing the binding of the cellular p53 protein to E6. This was followed by experiments showing that this binding promotes the degradation of p53 [128] mediated by the cellular ubiquitin proteolysis system [129,385]. p53 acts as an transcriptional activator by binding to specific DNA sequences [130] and is required for the growth arrest following cellular DNA damage [131,132]. Cells without functioning p53 are not arrested appropriately in G1 and display genomic instability [133,134]. The transcriptional activation by p53 induced after DNA damage is inhibited by HPV18 E6 [135].

The interaction of E6 with p53 is obviously the prime cause of chromosomal instability in cells infected by high risk HPVs [136–138]. It sensitizes human mammary epithelial cells to apoptosis induced by DNA damage [139]. In addition, the abrogation of the p53 function by transfection with the HPV16 E6 gene enhances the resistance of human diploid fibroblasts to ionizing radiation [140].

Degradation of p53 by E6, though apparently responsible for chromosomal instability and therefore presumably one of the main risk factors in the progression of premalignant lesions (see below), is not the sole function of this viral oncoprotein. The degradation of p53 seems not to be sufficient for a growth-stimulatory effect of E6 observed in human embryonic fibroblasts [141]. Moreover, the E6 protein of HPV16 functions as a transcriptional repressor of the Moloney murine leukemia virus long terminal repeat and of the cytomegalovirus immediate early promoter [142].

In addition to binding p53 and the cellular ubiquitin E6-AP, E6 interacts with various other, yet poorly defined cellular proteins [143] and with a putative calcium-binding protein [144].

4.5.2. E7 protein

The HPV16 E7 protein represents a zinc binding phosphoprotein with two Cys-X-X-Cys domains composed of 98 amino acids. A zinc binding domain and two Cys-X-X-Cys motifs reveal similarity to the E6 protein, suggesting an evolutionary relationship between the two proteins. The amino terminal part of the E7 protein contains two domains corresponding partially to the conserved region 1 (CR-1) and completely to conserved region 2 (CR-2) of adenovirus E1A proteins and to an analogous region in SV40 large T antigen [145]. Both of the E1A regions are involved in cell transformation [146]. Both corresponding domains in E7 (cd-1 and cd-2) contribute to the immortalizing potential of E7 [147]. In a yeast two-hybrid system dimerization of the E7 oncoprotein has been demonstrated in vivo [414].

Similar to E1A and SV40 large T antigen, high risk HPV E7 proteins complex with the retinoblastoma susceptibility protein pRB [148–150]. The binding affinity of high risk HPVs E7 for pRB is approx. 10-fold higher than that of low risk HPVs [384]. This difference results apparently from a single amino acid modification at the position 21 [151] which also influences the ability of E7 to cooperate with an activated *ras* gene in transformation of baby rat kidney cells [152]. pRB-binding, however, does not emerge as a general precondition for immortalization [153], pointing to additional functions of the E7 protein. In correspondence to E1A/pRB complexes, E7/pRB binding releases the transcription factor E2F from pRB complexes, activating transcription of genes regulating cell proliferation [154,155]. Interestingly, a strong RB-binding activity has also been reported for the low risk HPV1 virus E7 protein [156] which fails, however, to reveal other transactivating activities.

Besides binding pRB, E7 proteins of high risk viruses associate with related proteins, such as p107 and p130, and with the protein kinase p33cdk2 and with cyclin A [157,158]. Recent studies demonstrate that E7 expression in NIH3T3 cells results in a constitutive expression of cyclin E and cyclin A genes in the absence of external growth factors [159]. E7 activates the cyclin A promoter via an E2F binding site. Cyclin E activation requires the cd-2 domain, but not cd-1, whereas cyclin A activation

requires both domains. The cyclin E activation precedes that of cyclin A. Obviously E7 overrides two inhibitory functions restricting expression of cyclin E and cyclin A genes. Cyclin D1 expression is not affected by E7. The analysis of E7 mutants indicates that the activation of cyclins E and A cosegregates with the ability of E7 to transform [159]. Similarly, transcriptional activation by HPV16 E7 has also been reported from the adenovirus E2 [160,161] and B-myb promoters [162].

Recently it has been demonstrated that the HPV16 E7 protein can complement functions of E1A provided by the E1A aminoterminus and required for stimulation of adenovirus type 5 early promoters [163]. Under these conditions the association of protein complexes containing c-jun with ATF sites is stabilized. In addition, by using a glutathione-S-transferase fusion protein system, E7 complexes with AP-1 transcription factors have been demonstrated including *c-jun*, *jun-B*, *jun-D* and *c-fos* [415]. Mutational analysis revealed that the E7 zinc-finger motif, but not the pRB binding domain were involved in these interactions. Since a transcriptionally inactive *c-jun* deletion mutant also bound E7 and interfered with E7-induced immortalization, the *jun*-E7 interaction appears to be physiologically relevant.

High risk E7 proteins, to a lesser extent than E6 proteins (see below), can bypass DNA-damage p53-induced G_1 growth arrest [164–166,136], as a potential mechanism for the reported E7-induction of chromosomal aberrations [167]. The mode of this interaction is presently not fully understood, but suggests an interconnection between p53 and RB regulating pathways.

5. Transmission and natural history of papillomavirus infections

Transmission of human papillomaviruses is facilitated by the presence of abraded or macerated epithelial surfaces [168]. Anogenital infections are mainly transmitted by sexual contact. HPV DNA is rarely detected in sexually unexperienced young women [169–172]. There exists a correlation between the number of sexual partners and the prevalence of HPV infection [173–176]. Occasionally, anogenital HPV infections are also transmitted digitally from one epithelial site to the other [177,382]. They may be transmitted by fomites, by medical instruments and by laser plumes [178,179].

Oral-genital contact may lead to infections at oral sites by anogenital HPVs [180]. Salivary transmission probably accounts for additional infections of this region.

Skin infections by papillomaviruses originate from contacts with contaminated materials, walking barefoot on an abrasive surface [181,182] or by acquiring accidental epithelial wounding with contaminated equipment [183].

The natural history of HPV infections is presently not fully understood. It appears that the majority of these infections does not lead to visible lesions, it may be in fact abortive or is cleared by the immune system within a short period of time. Indeed, severe impairment of immune functions results in a high prevalence of clinically apparent HPV infections [184–187]. Obviously, infections with HPVs found almost exclusively in a rare hereditary condition, epidermodysplasia verruciformis (EV) (see below), must also be spread within the non-EV-population, since

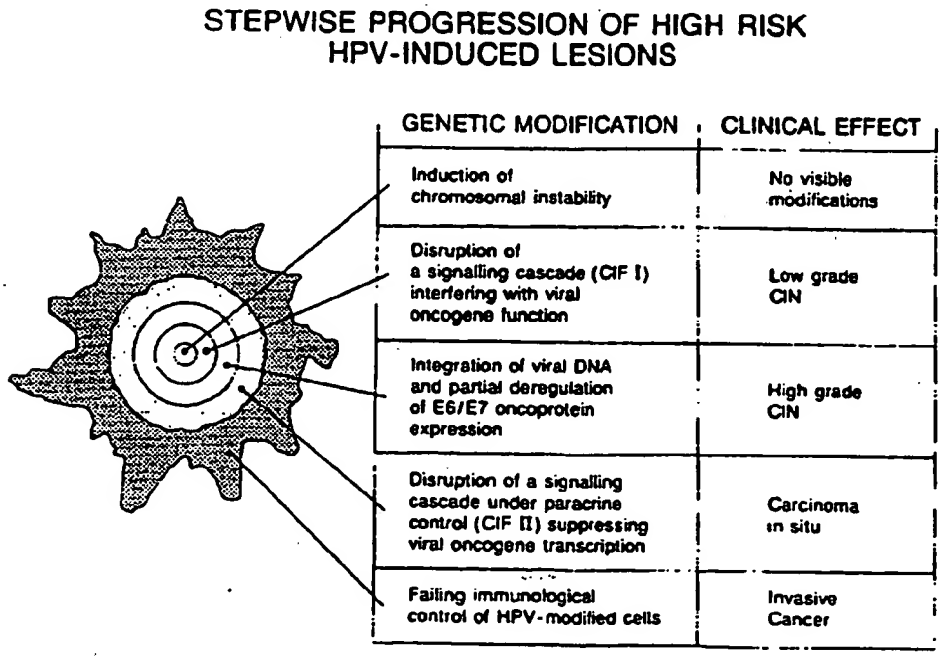

Fig. 2. Stepwise progression of high risk HPV-induced lesions. The inner circle schematically represents initially infected cells, subsequent circles symbolize progressively modified clones with increased expansion and growth potential.

individual cases of EV have been noted worldwide (see review [188]).

An important, yet unresolved issue is the clonality of intraepithelial lesions and warts. The existing literature on this topic is scarce and controversial [189,190]. A clonal growth of HPV-infected tissue may suggest that the development of lesions required already a highly specific intracellular environment, possibly only provided in cells with specific genetic modifications (selective gene inactivations or activations, mutations).

For anogenital HPV infections a schematic view of the progression of lesions is depicted in Fig. 2, mainly based on experimental findings within various stages of progression. The percentage of initially high risk HPV-infected women developing cancer of the cervix at a later stage of life is low [191]. It should be still much lower in infections with low risk viruses.

Virtually nothing is known about the mode of viral genome persistence in unapparent, latent, infections. HPV DNA presence has been demonstrated in clinically symptom-free epidermal and mucosal sites of the cervix, the larynx or the skin [192–195]. The mode of maintenance of this DNA persistence is unknown.

6. Non-malignant proliferations induced by papillomavirus infections

Papillomaviruses cause a wide spectrum of cutaneous, mucocutaneous and mucosal proliferations (see [196]). There exist various forms of common warts with an interesting localization-specific pattern of virus-types: plantar warts contain, for instance, most frequently HPV1 [24], the myrmecia type of plantar warts is frequently linked with HPV 63 infections [197], hand warts contain most often HPV2 and HPV4 [23,24]. It appears that individual virus types induce a somewhat specific histopathology and differ in their growth-stimulating potential.

The wart, however, is not the sole cutaneous manifestation of infections by specific types of papillomaviruses: various HPV types have also been noted in Bowenoid lesions and other intraepithelial neoplasias [177,198]. Again, the development of premalignant changes regularly, though not exclusively, depends on the infecting virus type. Solar exposure of HPV infected sites or HPV infection of the sun-exposed skin may lead to syncarcinogenic effects even after infection with low risk viruses [198].

Anogenital HPV infections are found in either mucocutaneous or mucosal localizations. It appears that HPV6 and HPV11 which cause the vast majority of genital warts prefer mucocutaneous sites, since most active proliferation and virus production is noted in condylomata acuminata at external genital sites. These viruses are only rarely found in cervical infections. HPV16, less frequently HPV18, and several other anogenital HPV types which probably represent high risk infections, cause Bowenoid lesions at external genital and perianal sites (vulvar, penile, perianal and anal intraepithelial neoplasias). The same viruses, besides some additional low risk HPV infections, are responsible for cervical intraepithelial neoplasias [199]. A careful analysis recently revealed that more than 96% of these lesions contain identifiable HPV types [35].

Oral lesions may contain anogenital HPV types, very regularly HPV11 or HPV6 are found in laryngeal papillomatosis [30]. Oral papillomas frequently contain these or other anogenital types [64,200,201]. Very few types have been thus far exclusively detected in the oral mucosa, besides HPV13 [202] and HPV32 [203] two new types were recently isolated from oral lesions of immunosuppressed patients. HPV 72 and HPV 73 [201].

It is likely that the vast majority of HPV infections remains clinically without symptoms or produces unapparent microlesions. The wide distribution of HPV types throughout the world, even of those found only in rare clinical conditions, like epidermodysplasia verruciformis, strongly suggests an effective mode of spreading within the human population and probably long periods of persistence and virus particle shedding from infected individuals. Though not stringent, there seems to exist a certain degree of specialization of subsets of HPV types to specifically differentiated human cells which either permit efficient infection or provide optimal conditions for viral particle maturation. The adaptation to these tissues may in part explain the development of the surprising heterogeneity of the papillomavirus group in the course of evolution within the same host.

7. Papillomaviruses causing cancer: general considerations and mechanistic aspects:

7.1. General considerations of causality

Although an infectious etiology of cancer has been suspected by some investigators for more than one century (reviewed in [204], and first animal data on cancer induction by animal viruses became available approximately 90 years ago [205,206], it proved to be exceedingly difficult to demonstrate a causal involvement of infections in human cancers. Several reasons account for this difficulty [207]:

Infections suspected to be involved in human cancerogenesis are frequently ubiquitous (e.g., Epstein-Barr virus, papillomaviruses, hepatitis viruses). Only a small percentage of infected individuals develops the respective form of cancer.

The time periods elapsing between primary infection and cancer development are frequently in the order of several decades.

The arising tumors are commonly monoclonal and, thus, cannot be the result of a systemic infection.

Chemical and physical cancerogens are frequently suspected to be causally related to the same tumor types.

These notions appear to be incompatible with cancer development as the immediate or sole consequence of an infection. The infection still could be necessary, but should be not sufficient for cancer induction.

One additional aspect adds to these difficulties: viruses may contribute to cancerogenesis by very different modes of interaction. Besides viral infections which seem to exert a growth-stimulating effect by *direct* and continued interactions with infected host cells (e.g. viral genome latency with continued expression of viral oncogenes, *cis*-activation of cellular genes due to viral DNA integration), other infections contribute to cancerogenesis *indirectly* (reviewed in [208]. Human immunodeficiency viruses (HIV) substantially elevate the risk for specific cancers (B-cell lymphomas, Kaposi sarcomas, non-melanoma skin cancers). Other virus infections, particularly with members of the herpes virus group, induce mutations in host cell DNA even after abortive and transient infections [209] and amplify under the same conditions the DNA of other DNA tumor viruses persisting in the same cells [210–212].

Papillomaviruses infections contribute directly to carcinogenesis, since (with the exception of one animal system [213]) viral DNA persists in the malignant cells and is genetically active [32,76]. Therefore, the following discussion will be limited to the potential causal link between these infections and human cancers and to *trans*-functions involved in cancer development.

In conventional infections Koch's postulates [214], with minor modifications, have been used effectively to establish causality. The propagation of the suspicious agent under experimental conditions outside the host and the induction of the respective disease in suitable experimental animals after prior in vitro cultivation are not applicable to many suspected human tumor viruses. Most of those cannot be propagated under experimental conditions nor are they tumorigenic for laboratory animals.

To overcome these problems, Evans [215] stressed seroepidemiological data as additional parameter to establish relationship between virus infections and specific human cancers. His suggestions were motivated by seroepidemiological data linking Epstein-Barr virus to specific human cancers (see review [216]). In papillomavirus infections none of these parameters holds up as valid: besides the absence of effective in vitro replication systems and suitable animal hosts, even seroepidemiology provides insufficient information. A substantial percentage of cervical cancer patients carrying HPV16- or 18-positive tumors appears to be devoid of detectable immune responses to antigens of the respective virus [217,218]. The now emerging possibility of seroepidemiological tests by using virus-like particles [42,43] may permit a more detailed analysis of the immunological response against HPV infections.

In spite of these problems, are there criteria available permitting an unequivocal establishment of causality for these infections? Indeed, this has been attempted previously [219] and is summarized as follows:

Epidemiological evidence (risk assessment, coincidence of geographic prevalence, seroepidemiology, plausibility of relationship) that the respective infections represent risk factors for the development of specific tumors;

Regular presence and persistence of nucleic acid of the respective infectious agent in cells of specific malignant tumors;

Stimulation of proliferation upon transfection of the respective genome or parts thereof in corresponding tissue culture cells;

Demonstration that the induction of proliferation and the malignant phenotype of specific tumor cells depend on effects or functions exerted by the persisting DNA of the infectious agent.

The most convincing criterium originates from experiments where the genetic activity of the latent viral genome has been knocked out in cervical carcinoma cells. As the consequence, a decreased proliferation rate and loss of the malignant phenotype have been demonstrated [49,220,221].

The application of these criteria appears to be useful for those viral systems where position effects of viral DNA integration or specific viral gene functions are suspected to be involved in cancerogenesis.

7.2. *High and low risk HPVs*

The original definition of specific HPV types as high risk viruses was based on their frequent presence in cervical and anogenital cancers [117]. In subsequent years the assignment of different properties to both groups of agents permitted a focussing of this definition. This became apparent when 'high risk' viruses were shown to immortalize human keratinocytes [222,223], whereas low risk viruses failed to do so. The subsequent observations on p53 and pRB binding by high risk HPV oncoproteins [127,150], in remarkable contrast to several low risk viruses, seemed to contribute another functional parameter for this differentiation. The induction of chromosomal aberrations as the consequence of high risk viral oncoproteins overriding cell cycle control mechanisms [224,164–166,136] emerges as the functionally most important distinction between these virus groups. Due to these properties high risk viruses are able to directly contribute to the progression of latently infected cells and may act as solitary carcinogens [208].

Cancer cells containing low risk HPV genomes frequently reveal modifications in the cellular p53 gene and occur at sites exposed to chemical or physical carcinogenic factors. Basal cell and squamous cell carcinomas of the skin (see below) are one example, extensive laryngeal papillomatosis which had been X-irradiated in past decades and converted subsequently into squamous cell carcinoma of the larynx (summarized in [405]) represent another one. Although the actual role of low risk HPVs in these malignant conversions is not yet clarified, the observations

suggest that mutagenic modifications of host cell genes, presumably required to activate the oncogenic potential of these viruses, are mediated in these instances by the physical carcinogens. The apparent inability of these viruses to code for mutagenic oncoproteins seems to be the main reason for their failure to act as solitary carcinogens and for their dependence on interaction with other mutagenic factors in the generally rare events of malignant development following these infections.

8. Immortalization of tissue culture cells by papillomaviruses

Immortalization of tissue culture cells by viruses is defined by the induction of continuous growth in vitro without detectable tumorigenicity of these cells after heterotransplantation into immunosuppressed animals. Transformation, in contrast, defines continuous growth of cells which, upon heterotransplantation under the same conditions, form invasively growing tumors.

The definition of immortalization is derived from experimental conditions. It is not entirely clear how it correlates to clinical HPV-induced lesions. It is, however, suggestive that it corresponds at least to a proportion of low grade intraepithelial neoplasias, as deduced from three types of observations: immortalized cells in organotypic cultures histologically resemble low grade intraepithelial lesions (reviewed in [225]. In addition, clinical low grade neoplasias, in contrast to high grade lesions and invasive cancer, reveal a similar restriction of E6/E7 oncogene transcription, as do immortalized cells, when the latter are heterografted into immunocompromised animals [79]. Finally, though difficult, it has been possible to cultivate immortalized lines from explants of intraepithelial neoplasias [226–228].

Early attempts to immortalize cells by papillomavirus infection date back to 1963 when Black et al. and Thomas et al. demonstrated immortalization of fetal bovine cells by bovine papillomavirus infection. In 1980 Lowy and colleagues [393] showed that only 69% of the genome was required for successful immortalization.

The first reports on attempts to immortalize or transform murine cell by human papillomavirus types appeared in 1984 and 1986 [229,230]. This was quickly followed by similar data after transfection of rat cells with HPV16 or HPV18 DNA [231,232]. At about the same time it became apparent that the HPV16 E7 gene cooperates with the *ras* oncogene in the transformation of primary rat kidney cells [233,161].

Immortalization of human cells was with HPV16 DNA was first achieved in 1987 [222,223] and with HPV18 DNA in 1988 [237]. Subsequently a large number of additional human cell types, including skin, bronchial and kidney epithelium, smooth muscle and endothelial cells, have been immortalized by high risk HPV DNA transfection (reviewed in [225]). Recent reports describe the immortalization of human prostate [234] and ovarian cells [235] by high risk HPV DNA. Only DNA fragments carrying the E6/E7 genes were necessary for immortalization of rodent [231] and human cells [236,237,47].

Although the expression of E6/E7 genes is necessary for immortalization of cells by high risk HPV, this expression is clearly not sufficient [238]. There exists good evidence for the need of modifications in specific host cell genes as an additional prerequisite for immortalization. This has been demonstrated by somatic cell hybridization studies, initially performed with cells from other virally immortalized lines [239,242,240], subsequently also with HPV-immortalized cells [241]. Only a small fraction of initially infected or transfected cells eventually becomes immortalized, the vast majority of these cells continues to express viral oncoproteins [242] or, in the case of HPV transfection, continues to transcribe E6/E7 message [241] and undergoes senescence.

Somatic cell hybridization performed with different clones of immortalized cells led to the identification of four complementation groups complementing each other for senescence. Although this number may increase in the future, it suggests that the failure of each one of at least four cellular genes, presumably engaged in the regulation of the same signalling pathway [57], in addition to viral E6/E7 gene expression may result in immortalization.

The involvement of cellular genes whose failing function in the presence of HPV oncogenes leads to immortalization points to two predictions: the function of these genes in non-modified cells interferes with the function of viral oncoproteins, as evidenced by the continued expression of the latter even in cells undergoing senescence. Secondly it leads to the expectation of specific mutational changes, possibly even visualized by specific chromosomal aberrations, in immortalized cells. There exist indeed first reports on specific chromosomal aberrations in HPV-immortalized human keratinocytes, preferentially involving sites on chromosomes 3 and 18 [243–245,390]. The interpretation of these data is still difficult, since some of these immortalized cells had converted to malignant growth. It seems to be relevant, however, that even malignant HPV-positive cells can be converted to senescence by the introduction of chromosome 11 [246–249] chromosome 4 [250] chromosome 2 [251] and chromosome 1 [252]. Unfortunately, most of these studies, particularly those on chromosome 11, and 2, were performed with malignant lines, rendering an interpretation of the relationship of these suppressing events strictly to immortalization impossible.

Cellular genes engaged in the prevention of virus-induced immortalization have not yet been identified. It has been speculated that one of these genes may code for the cyclin-dependent kinase inhibitor $p16^{INK4}$ protein [253]. This protein is upregulated under conditions of pRB inactivation [254–256]. Since the pE7/pRB interaction results in pRB inactivation (see Section 4.5 on E7), the upregula-

tion of $p16^{INK4}$ may account for the growth limitation of primary HPV-infected cells resulting in senescence after a prolonged lifespan. This could provide an explanation for the functional impairment of senescent HPV oncoprotein-expressing cells. In spontaneous immortalization of Li-Fraumeni syndrome fibroblasts loss of $p16^{INK4}$ expression has been consistently observed [257]. A very recent study suggests that loss of $p16^{INK4}$ may be required for the immortalization of human uroepithelial cells by HPV 16 E6, but not for those immortalized by E7 or jointly by E6/E7 [258]. More studies need to be done on $p16^{INK4}$ expression in HPV-immortalized cells to clarify the role of this gene product and of other cyclin-dependent kinase inhibitors in the suppression of immortalization.

It is unlikely that p53 is directly involved in the control of immortalization. This can be deduced from experiments in mice where a p53 knock-out phenotype results into a relatively normal development of these animals with an increased tumor incidence in later life [259]. It is however highly likely that p53 plays a very important indirect role for the progression of HPV infected cells towards immortalization and even to a malignant phenotype [208]: the inactivation of p53 by the E6 protein emerges as the responsible event for the prevention of the p53-mediated G1 arrest following DNA damage [224,136]. Accumulation of resulting mutations in the course of subsequent cell divisions is probably a most important precondition for the eventual selection of cell clones which acquired mutations in cellular genes controlling immortalization. *The functionally inactive p53 thus seems to represent the most important progression factor*, possibly without a direct role in immortalization and transformation.

It has been speculated that the stability of telomere sequences regulated by the telomere polymerase plays an important role for cell proliferation and senescence [260]. Indeed, telomere shortening is consistently observed under conditions of cellular senescence [378], whereas activation of telomerase and recovery of telomere length with telomere stabilization occurs in immortalized cells [261]. Telomere shortening is also observed in normal human and pre-crisis HPV-expressing cells with a recovery of telomere length after immortalization [391]. A recent study showed that telomerase becomes activated by the E6 gene product of human papillomavirus type 16 even in keratinocytes which do not become immortal after E6 introduction [262], indicating that telomerase activation is insufficient for immortalization.

Hormones may play an important role in in vivo events related to immortalization (see below) of HPV infected cells. High risk HPVs harbor glucocorticoid responsive elements within the long control region [263]. Glucocorticoids enhance substantially immortalization by HPV 16, but fail to induce the same activity in HPV 11 infections [264,265]. The hormone-dependent transformation by HPV 16 and ras can be inhibited by a hormone antagonist RU 486 [266]. Although all these studies were performed under tissue culture condition, it is suggestive that reported marginal increases in the risk for cervical cancer development under long-time oral contraceptive may be related to these observations [267,268].

9. Malignant progression

Numerous studies indicate that there exists a substantial time lag between primary infection by high risk HPVs, development of cervical intraepithelial neoplasias, carcinomata in situ, and finally invasive cancer. Anogenital HPV infection occurs most frequently at young age with the onset of sexual activity and depending on the number of sexual partners. High rates of HPV detection have been reported in females in Western countries in age groups between 16 and 20 years [193], summarized in [388]. Cervical intraepithelial neoplasias reveal a peak incidence in age groups between 25 and 35 years, whereas cervical cancer incidence peaks between 55 and 65 years of age [191]. This suggests already that the latency period between primary infection and development of intraepithelial neoplasia averages several years, that the progression towards invasive growth seems to require additional 20 to 30 years [207].

The molecular basis for this long time span becomes increasingly understood: in situ hybridization studies [79,98] and analyses of HPV16 E6/E7 transgenic mice [269] indicate an incremental upregulation of HPV E6/E7 expression at each stage of neoplasia. Inversely, downregulation of E6/E7 transcription takes place in HPV-immortalized non-malignant cells after heterografting them into immuno-compromised animals [270,271] or by exposing them to human macrophages in vitro [272]. This results at the same time in a marked growth inhibition. Finally, the selective inhibition of HPV18 E6/E7 gene expression in cervical carcinomas cells results in growth inhibition and loss of tumorigenicity [220,49]. All these data show that high risk HPV E6/E7 expression is a prerequisite for continued growth stimulation. Its upregulation in the course of progression suggests a correlation between the quantity of the viral oncogene product and the severity of the lesion. The selective E6/E7 downregulation after heterografting non-malignant cells into immuno-compromised animals shows, moreover, that the regulation of viral oncogene expression differs between immortalized and malignant HPV-harboring cells.

10. The CIF-concept

In Section 8, the role of the failure of a controlling signalling cascade *interfering with the function* of high risk HPV oncoproteins for the development of the immortalized phenotype has been discussed. Thus, immortalization requires viral oncogene expression, but, in addition,

the functional failure of alleles regulating this signalling cascade. The reasons for suspecting a proportion of low grade cervical intraepithelial neoplasias as correlate of a similar in vivo failure were also summarized there. Malignant progression obviously depends on additional changes within the genome of the infected cell. As pointed out in the preceding paragraph, apparently an additional signalling cascade needs to be interrupted, *interfering with the transcriptional activity* of the viral oncogenes. The existence of a cellular interference factor (CIF) was initially postulated in 1977 [273,207,406]. The emerging picture today points to the existence of *two CIF-cascades*, whose sequential interruption in an HPV-positive cell is the precondition for HPV-linked cancers.

Early evidence for the existence of a cellular control of viral oncogene transcriptional regulation originated from studies revealing a selective downregulation of HPV transcription in non-malignant HeLa-fibroblast hybrids by 5-azacytidine treatment in contrast to parental HeLa cells [274]. Similarly, the HPV promoter, regulating the transcription of HPV oncogenes in cervical carcinoma cells, was silenced after subjecting these cells to somatic cell hybridization with normal keratinocytes [275]. The downregulation of E6/E7 transcription in non-malignant cell hybrids and immortalized cells after heterografting them into susceptible animals [270,79,98] further supported this interpretation. These experiments pointed to a paracrine regulation of viral oncogene transcription. The demonstration of an HPV transcriptional repressing effect of human and murine macrophages [272] and unpublished) and of specific cytokines excreted by activated macrophages, like TNFα and Interleukin-1 [276,394,272] and TGFβ [277], suggests a cytokine-mediated response to paracrine signals.

Individual steps of this signalling cascade still have to be elucidated. Besides specific cytokine receptors, it is likely that a region in the short arm of chromosome 11, apparently engaged in the regulation of protein phosphatase 2A (PP2A), plays an important role: deletions in this region lead to an upregulation of a regulatory subunit of PP2A, enhancing HPV transcription in human fibroblasts [278]. Similar effects have been noted after inhibition of the catalytic subunit of PP2A by ocadaic acid or SV40 small t antigen.

The terminal effect of this signalling cascade should be mediated by specific transcription factors. A number of these factors have been shown to be important for the regulation of HPV transcription (reviewed in [53]. A number of negative regulators have been identified: the Oct-1 transcription factor [53], the nuclear receptor for Il-6, NF-Il-6 [276], retinoic acid receptors [279–281] and YY-1 [56]. Except for Oct-1, the other factors show some differential activity in non-malignant when compared to malignant cells. YY-1 interacts with an upstream 'switch'-region in HPV promoter suppression [282].

Among factors positively regulating the HPV promoter (see review [53]), AP-1 seems to play a particularly important role [283,284,52]. Modification of the proximal AP-1 binding sites results in a loss of transcriptional activity. Recent data indicate that in the course of antioxidant or TNFα-induced transcriptional inhibition of E6/E7 transcription the composition of AP-1 complexes changes [401]. C-*jun* and *jun-B* heterodimerize with *fra-1*, the synthesis of these proteins is substantially enhanced in non-malignant cells under such conditions. In spite of an increased synthesis as well of *c-fos*, this protein is no longer found in AP-1 complexes and may be rapidly degraded. Thus, the upregulation of *fra-1*, probably triggered by cytokine interaction, and its increased presence in AP-1 heterodimers could represent one of the decisive factors in the differential control of HPV transcription in non-malignant cells. *Fra-1* is not upregulated in the malignant cervical carcinoma cells tested thus far.

The disruption of cellular signalling cascades resulting from modifications of host cell DNA sequences requires mutational events or epigenetic modifications (e.g., methylation) of cellular DNA. In high risk HPV infections this is most likely mediated by mutagenic activity of the E6 and to a lesser degree the E7 oncogene expression (see Section 4.5.1 and Section 4.5.2). Continued expression of these oncoproteins, besides their inherent growth-stimulatory activity, will gradually lead to a selection of cell clones with specific mutations, permitting further dysregulation of viral oncogene expression and viral oncogene activity. A schematic representation of these events is outlined in Fig. 2. Thus, high risk HPVs seem to act as solitary carcinogens.

11. Hereditary factors in papillomavirus susceptibility and carcinogenesis

Persistence and appearance of papillomavirus-induced lesions is obviously influenced by genetic factors, at least in a number of conditions: The genetic predisposition for epidermodyslasia verruciformis has been discussed before. Similar data exist for focal epithelial hyperplasia of the oral mucosa [285–287] which occurs at high frequency among American Indians and in Eskimos.

For squamous cell carcinomas of the cervix a high risk has been reported in women with HLA DQw3 [288]. Although these data have been disputed in additional publications [289,290], they are supported by similar observations in rabbit systems [291].

It appears to be very likely that our increasing knowledge of mutations of specific cellular genes, controlling HPV oncoprotein function or viral oncogene expression, will lead to the identification of a higher number of individual genes whose failure will predispose to cancer development. The existence of these modified genes within the germ line will depend on the compatibility of these modifications with the development and survival of the affected embryo.

12. Role of viral DNA integration

It has been noted early after the identification of HPV DNA in cervical cancer that the viral DNA regularly becomes integrated into host cell DNA [32,76,292,398]. The integrational events results in an interruption of the E2, frequently also of the E1 open reading frames [76] and to deletion or partial deletion of the downstream genes E4, E5, and L2. The E2 protein codes for transcriptional regulatory proteins that can both activate and suppress viral transcription [73,293]. It has been shown that the disruption of this intragenomic regulation leads to dysregulation of viral E6/E7 transcription and increases the immortalization capacity of the HPV16 genome [77]. The integration also leads to an increased stabilization of the viral message, apparently by inactivating a destabilizing element in the 3'-region of the viral mRNA. This stabilization is resulting from the chimaeric composition of E6/E7 message with flanking host cell sequences [76].

Viral DNA integration and dysregulation of E6/E7 oncogene activity is clearly not sufficient for the development of a malignant phenotype. Integration of viral DNA has been noted in a number of high grade intraepithelial lesions [294,295]. Moreover, somatic cell hybridization of cervical carcinoma cells with normal cells regularly leads to a non-malignant phenotype, inspite of the continued presence and expression of integrated viral DNA [270]. Finally, in up to one third of cervical biopsies exclusively episomal persistence of viral DNA has been noted [78].

Integration adds, however, to the dysregulation of viral oncogenes. The most likely mode of this dysregulation is probably less an increase in transcriptional activity rather than the increased stability of E6 and E7 mRNA as a result of the disruption in the 3' untranslated part of the early viral region. The A + U-rich element within this region confers instability on a heterologous mRNA [296]. Indeed, a higher level of E7 protein has been found in clonal cell populations of human cervical epithelial cells containing integrated HPV 16 DNA when compared to those containing exclusively extrachromosomal viral DNA [297].

13. Specific chromosomal aberrations in cervical cancer

The prediction of interrupted cellular signalling cascades in the development of cervical cancer should stimulate studies to identify specific chromosomal aberrations in these malignant, but also in premalignant lesions. Nonrandom structural changes and numerical chromosome aberrations have been reported in cervical cancer [298–300]. Loss of heterozygosity (LOH) was most frequently observed in chromosome arms 6p21-23, 3p13-25, and 18q12-21. A number of other loci with LOH were identified on 16 additional chromosome arms, with higher frequency for chromosomes 11p and 11q and chromosome 17p [300]. A rearrangement of chromosome 11q13 has been observed in three cervical carcinoma cell lines [301]. It may be of substantial interest that this chromosomal region also harbors the fra-1 gene [302]. A gene (HTS1) in chromosome 11p15 was localized and identified as a tumor suppressor gene for HeLa cells [303].

Integration of viral DNA may also occur into specific chromosomal loci potentially engaged in the control of viral oncogenes resulting in the selection of such clones ([304] and unpublished data). The region 10q24 has been identified in these studies with LOH in several cervical carcinoma biopsies. Another integration site has been found in chromosome 12q14-15 in two cell lines (SW756 and SK-v) derived from genital tumors [413].

Specific genes modified in some cervical cancer biopsies involve the jun-B gene [305], the ETS2 oncogene [248] and the DCC gene [306]. Their role in HPV oncogene regulation remains to be established.

14. Papillomaviruses in human cancers

14.1. Papillomaviruses in cancer of the cervix and in other anogenital cancers

HPV 16, 18, and a number of additional HPV types have been found in about 95% of all biopsies derived from cancer of the cervix throughout the world, when testing was performed with a sensitive technology and screening permitted the detection of more than 4 HPV types [307,308,34]. HPV16 is by far the most frequently found virus type and accounts for 50 to 60% of all positive data. the presence of HPV18 varies between 10 and 20%. A number of analyses failed to include specific testing for HPV45, a type relatively closely related to HPV18. Resulting cross-hybridizations may have led to an overestimation of HPV18 positivity. Many of these types have been found in exceptional cases only.

The mere presence of HPV DNA in the vast majority of biopsies from cancer of the cervix does not prove an etiological involvement (see above). Early data pointing to a role were derived from experimental findings which have been discussed in the previous sections:

- the regular expression of HPV E6/E7 genes within the cancer cells,
- the absence of detectable cellular regulation of E6/E7 genes in cancer cells,
- the immortalization of human cells by the expression of these genes,
- E6/E7 protein induction of growth promotion and chromosomal instability,
- cessation of cell growth and reversion of the malignant phenotype in cells of cervical carcinoma cell lines after selective blocking of E6/E7 gene function.

These data essentially demonstrate that the E6/E7 oncoproteins are the main determinants of the malignant phenotype in those cervical carcinoma cells which have

been tested thus far. They provided the background for epidemiological studies analyzing the role of HPV in cancer of the cervix.

Case-control studies contributed mainly to the epidemiological link of HPV infections to cancer of the cervix. Although initial studies suffered from problems related to test validity and study design [309,310], later studies were better controlled for known or suspected risk factors for cervical cancer (summarized in [388]). In a careful population-based case-control study [32] analyzed 436 incident cases of squamous cell invasive cancer and 387 population controls in a low risk country (Spain) and a high risk country (Colombia). The odd ratios (OR) for HPV DNA were 46.2 in Spain and 15.6 in Colombia. Among HPV-negative cases, the risk factors identified were related to sexual behaviour. In the HPV-positive group the only other recognizable risk factor (besides HPV) were the use and duration of oral contraceptives intake [311]. The majority of 28 other studies came up with OR values between 10 and 50, five of these were below 5, five of them above 50 [388]. Two of the latter exceeded OR values of 100. By including only those studies which used PCR technology, the OR slightly exceeded 50 in average. Thus, the OR for anogenital HPV infections as risk factors for cancer of the cervix clearly and substantially tops those reported for long-time tobacco smoking and lung cancer [387].

Cohort studies have been performed to study the transition of HPV infection to cervical intraepithelial neoplasia (CIN) and the progression from CIN to cancer. They have been summarized previously [388]. A careful analysis by [312] revealed a relative risk (RR) of HPV-positive women for the development of high grade CIN of 11. All other available prospective data indicate that HPV16 and 18 infections precede high grade CINs and predict an elevated risk to develop these lesions (see [388]). Thus, epidemiological studies now strongly support the experimental data implicating specific HPV types as cervical carcinogens. The absence of significant other identifiable risk factors also supports the experimentally derived concept [208,386,399] that high risk HPV types may act as solitary carcinogens.

The combined sets of experimental and epidemiological data prove the etiological role of specific HPV types in a high proportion of cervical cancers. The presence of additional types in cervical carcinoma biopsies renders it likely that they also play a decisive role in the development of these cancers. It is presently an open question whether the remaining approximately 5% of 'virus-free' cervical cancers are indeed not harboring viral DNA, and thus may have been caused by other factors, or whether they contain yet not identified novel HPV types. Although it is likely that the majority of anogenital HPV infections has been identified by now, there still exists the possibility of additional infections.

In cancer of the vulva a number of studies reported a lower incidence of HPV positivity when compared to cervical cancer [388]. Sensitive studies which included testing for more types than HPVs 6, 11, 16, and 18 reported positive findings between 30 and 86% [313–315], with an overall positivity slightly above 50%. There appear to exist two different types of vulvar cancer: squamous cell carcinomas affecting a younger age group, preceded by vulvar intraepithelial neoplasia, containing HPV DNA (most frequently HPV16); and another group occurring at higher age with lichen sclerosus-like lesions devoid of detectable HPV DNA [316,317]. It will be interesting to analyze tumors of the latter group for those cutaneous HPV types which have been recently found in squamous cell carcinomas of the skin in immunocompromised and immunocompetent patients [198].

The number of vaginal cancers tested for HPV DNA is still small. 52 cases have been reported in the literature, tested under conditions of different sensitivity and specificity. 35 of these biopsies were tested by procedures which permitted HPV typing beyond the four standard types. Twenty of these (57%) turned out to be HPV-positive [318,319,389]. Since the number of types analyzed in these tests was still very limited, this percentage of positivity most likely represents an underestimate of the real figure.

In penile cancer, the situation appears to resemble that of vulvar cancer, although the overall rate of HPV positivity seems to exceed that of the latter [388]. Studies which included a larger spectrum of types ranged in their positivity between 54 and 100% [320–323] with an average of 73% positivity. Most frequently again HPV16 is detected in these tumors. The total number of tumors tested under sensitive and specific conditions is here very limited, too. Yet, there may also exist an entity of penile cancers without the DNA of anogenital HPV types.

The rare verrucous carcinomas of penis or vulva and Buschke-Löwenstein tumors frequently contain HPV6 or 11 DNA [324].

In anal and perianal cancer the limited number of available studies describe a high degree of HPV positivity, exceeding 70%, when testing was performed under appropriate conditions [325,326]. Here the situation seems to resemble cancer of the cervix, although the limited number of tests does not permit firm conclusions. Again, the small number of HPV types analyzed in these biopsies renders it highly likely that the available detection rates represent underestimates.

In summary, papillomavirus DNA is detectable in at least 95% of cancers of the cervix and in more than 50% of vulvar, vaginal, penile, anal, and perianal cancers.

14.2. Papillomaviruses in non-melanoma skin cancers

Early studies on papillomaviruses in skin cancer were conducted in patients with epidermodysplasia verruciformis (EV) (reviewed in [188]. EV is a rare hereditary condition occurring worldwide, in which initially benign

papillomatous lesions arise frequently at the age of 5 to 8 years. Some of these lesions appear as red plaques and are often overlooked by the patients. In about one half of the patients the lesions progress within the following 20 to 30 years. At light-exposed sites initially actinic keratoses and Bowenoid changes develop. Cancer progression occurs mainly on the forehead and on traumatized sites, resulting in local destruction, usually without metastazation.

More than 20 individual types of HPV have been demonstrated in benign lesions of EV-patients. The same patient frequently reveals several HPV types in different lesions. Interestingly, squamous cell carcinomas developing in these patients usually contain HPV5, some of them HPV8, but rarely other HPV types [327].

Although specific HPV types have been found in squamous cell carcinomas of EV patients already in 1978, their causal role for the induction of these cancers, though suggestive, is not proven in a formal sense. This is due to the difficulties in obtaining EV HPV-positive cell lines and to the inability of these viruses to immortalize human cells. In view of the observations that most of the malignant lesions develop at light-exposed sites, it is likely that an interaction between sunlight exposure and HPV infection represents the major reason for cancer development. The exact nature of this interaction remains to be established.

The underlying genetic effect of predispositions to epidermodysplasia verruciformis is not yet clear. Interestingly, these patients do not show an increased sensitivity to anogenital HPV infections. It has been described that EV patients reveal an inhibition of natural killer cell activity [328] and of cytotoxic T cells [329]. As a constant abnormality, cutaneous anergy to strong contact sensitizers (dinitrochlorobenzene - DNCB) has been noted in EV patients [330]. EV-like lesions have also been observed in immunosuppressed patients following organ transplantation [331–334] or after infection with human immunodeficiency virus [335,336].

Early studies on non-melanoma skin cancers were mainly conducted by using probes specific for anogenital HPV types [337,338]. With the exception of periungual Bowen's disease and periungual squamous cell carcinomas [339,177,340–342] which regularly contain HPV 16 DNA, a low percentage of HPV positivity was noted (reviewed in [388]). One notable exception was the finding of HPV 41-positive cancers in 2 out of 10 squamous cell carcinomas [343].

Recently, the use of consensus primers covering a broad spectrum of HPV types changed the picture substantially: EV types [344] but also a larger number of novel HPV types [198] were discovered in about 80% of squamous and basal cell carcinomas of immunosuppressed patients. Even in immunocompetent patients, approximately 30% of these tumors contained identifiable DNA, when tested with 16 different combinations of consensus primers [198]. A large number of different HPV types have been found in these tumors without a prevalence of specific types. This is a rather puzzling result and presently difficult to interpret. Since these types of tumors again develop almost exclusively at sun-exposed sites, an interaction between these virus infections and solar UV-irradiation could represent an explanation. Probably all these virus types represent low risk infections and are non-mutagenic for host cell DNA. Only additional mutations induced by sun exposure may result in malignant progression. This, of course, requires further investigation.

The present unavailability of suitable in vitro systems to analyze the biological activity of cutaneous HPV infections contributes to the difficulties in assessing their role in skin tumor progression.

There exist no reports on extensive testing of melanomas for HPV DNA. One positive finding of HPV37 in one melanoma remained solitary up to today, the same study failed to find this DNA in 35 additional melanomas or in 190 other skin tumors [345].

14.3. Papillomaviruses in cancers of the oral cavity, the larynx, the lung and the nasal sinuses

Cancers of the oral cavity, the tongue, the hypopharynx, and the larynx have been mainly analyzed for anogenital and cutaneous HPV infections. The vast majority of identified HPV types originates from these two sites, only very few specific types have been isolated from the oral cavity [202,226,201]. In view of the frequent occurrence of papillomas or papilloma-like lesions in the oral cavity [346], reviewed in [347], among them a fair percentage without HPV DNA detectable by the probes used in these studies, it is likely that careful screening will lead to the discovery of additional papillomavirus types, specifically infecting these mucosal sites.

The first reports on the presence of HPV in malignant tumors of the oral cavity appeared in 1985 [348,349]. They were followed by similar reports on HPV DNA in individual cases of cancer of the larynx [350–352]. Although subsequently, by using PCR analysis, the HPV detection data varied between 0 and 100% (see [388]), studies performed with reliable techniques commonly came up with positivity rates between 10 and 20%. In these studies most frequently HPV16 has been found, HPV18, HPV6, HPV11, and HPV2 were also reported in individual biopsies. One specific form of oral cancer, tonsillar carcinomas, revealed DNA of anogenital HPV types at exceptional frequency, exceeding 50% of the biopsies tested [353–355].

In laryngeal carcinomas the situation resembles that of other oral cancers. Again a wide variety of data has been published ranging between 0 and 100% (see [388]). It appears, however, that the situation is not different from other oral cancers, in that an average of close to 20% is presently positive when tested by reliable procedures. Again most reported data preferentially included anogenital types as probes.

Patients with laryngeal papillomatosis with subsequent development of laryngeal or lung cancer contained HPV6 or 11, and in one case HPV16 [356–359]. Other lung carcinomas were only exceptionally reported to be positive for HPV DNA [360,361]. Other studies failed to detect HPV DNA in this type of cancer [362,363]. By using a wide spectrum of consensus primers, [364] failed to find positive tumors in a larger series of these cancers. Thus, lung cancer with few exceptions seems to be devoid of presently detectable HPV genomes.

In carcinomas of the nasal sinuses, HPV 16 and 18 have been recorded (see [388]), in addition to HPV 57 which is more frequently found in inverted papillomas [365–367].

14.4. Papillomaviruses in cancers of the esophagus

A role of papillomavirus infections in cancer of the esophagus has been postulated initially by Syrjänen in 1982 [403], based on histological changes similar to those of condylomatous lesions in esophageal squamous cell carcinomas. Inspite of numerous attempts to clarify the role of HPV in this cancer (see [388]), the available data do not permit firm conclusions. The available studies are frequently based on in situ hybridizations or solely on PCR technology. A Southern blot hybridization study performed by [368] failed to provide evidence for positive tumors, although the same authors find close to 50% positivity by applying a PCR reaction specific for anogenital HPV types. In view of additional PCR data reporting positive results in the range between 7 and 24% [369–371], it is likely that a certain percentage of these tumors contains identifiable types of HPV genomes. A careful reappraisal of these data by using tests with a broad reactivity is clearly desired in order to elucidate the role of HPV in this frequent form of human cancer.

14.5. Additional cancers suspected to be linked to papillomavirus infections

Although a number of reports have been published claiming the presence of HPV DNA in human prostatic cancer, in cancer of the ovary, the colon, and cancer of the breast (see [388]), these studies have not been confirmed in other laboratories and remain at present unexplained.

Well-documented individual cases, however, exist in cancers of the bladder [372–374] and urethra [375,376,404]. The percentage of positive tumors in bladder cancer is usually low; in most studies below 10%. In urethral cancers higher rates of positivity have been noted. This is not unexpected since the latter site may be directly exposed to HPV infections.

15. The global role of HPV-linked cancers and conclusions

Cervical cancer represents the second most frequent malignant tumor of women worldwide with an estimated frequency of approx. 440000 new cases per year, corresponding to about 5.8% of the global cancer incidence [400]. If one considers that more than 50% of vulvar, penile, perianal and anal cancers, in all likelihood more than 20% of oral, laryngeal and nasal cancers contain predominantly anogenital high risk HPV types, this leads to almost 10% of the worldwide cancer burden linked to these infections.

Recent data point also to a role of low risk types in non-melanoma skin cancer, here in conjunction with a physical carcinogen, solar exposure. Non-melanoma skin cancer is the most frequent malignancy in caucasian populations. Although the latter data need to be substantiated, there is good reason to suspect that the actual contribution of papillomavirus infections to human cancers well exceeds the 10% mark. This identifies infections by certain members of this virus group as one of the most important risk factor for human cancer development. In view of rather excellent prospects of controlling at least some of these infections by vaccination [377], the identification of an HPV etiology of a major fraction of human cancers paves the way for new strategies in cancer prevention.

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